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Integration of the DNA of Mouse Mammary Tumor Virus in Virus-Infected Normal and Neoplastic Tissue of the Mouse

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Summary

We have used restriction endonucleases which cleave the DNA of mouse mammary tumor virus (MMTV) at one site (Eco RI) and at several sites (Pst I, Sac I and Bam HI) to study infection and mammary tumorigenesis in mice. Proviruses acquired during infection of BALB/c mice foster-nursed by virus-producing C3H females can be distinguished from the MMTV proviruses endogenous to uninfected BALB/c mice by the nature of the fragments generated with Pst I and Bam HI. Using this assay, we show that lactating mammary glands as well as mammary tumors from BALB/cfC3H mice have acquired MMTV DNA, and that a minimum of ~10% of normal glandular cells can be infected.

The new proviruses appear to be linked to cellular DNA of mammary tumors and infected lactating mammary glands within a limited region (0.2×10^6 daltons) of the viral DNA; the location of this region, based upon mapping studies with unintegrated MMTV DNA, suggests that the orientation of these proviruses is colinear with linear DNA synthesized in infected cells and thus approximately colinear with the viral RNA.

Comparisons of many mammary tumors and studies of lactating mammary glands with a high proportion of independently infected cells indicate that a large number of sites in the cellular genome can accommodate a new provirus; the acquired proviruses are rarely, if ever, found in tandem with each other or with endogenous proviruses. We cannot, however, distinguish between random integration and integration into a large number of preferred sites in the host genome.

Since Eco RI and Bam HI cleavage of DNA from each mammary tumor generates a unique set of viral-specific fragments, we propose that the tumors are composed principally of cells derived from a subset of the many infected cells in a mammary gland; this proposal is supported by our finding that Eco RI digestion of DNA from several transplants of a primary tumor yields the pattern characteristic of the primary tumor.

Introduction

The appearance of mammary tumors in most mouse strains is dependent upon infection by the mouse mammary tumor virus (MMTV), an enveloped RNA virus usually found in the milk of animals with a high tumor incidence (Nandi and McGrath, 1973; Bentvelzen, 1974; Moore, 1975). The natural history of the infection and the mechanism of the consequent tumorigenesis have, however, remained obscure. A detailed study of the molecular events that take place during replication of MMTV in infected cells has been possible only recently, since most cultured cells appear to be refractory to infection; however, the available evidence suggests that MMTV replicates in a fashion similar to other retroviruses, with integration of a DNA copy of the viral genome as a central step in the virus life cycle (Vaidya et al., 1976; Ringold et al., 1977b).

We are studying the natural history of infection by MMTV and attempting to understand its oncogenic activity by direct biochemical analysis of viral-specific macromolecules in tissues obtained from infected animals (Varmus et al., 1972, 1973) and in cells infected in culture. This report illustrates the use of restriction endonucleases for distinguishing MMTV-infected from uninfected tissues, and attempts to determine whether unique sites in viral DNA and cellular DNA are used during integrative recombination in the natural host. We have confined the experiments described here to one relatively simple biological situation in which we compare a BALB/c substrain infected by foster nursing on C3H mice (BALB/cfC3H) and the original uninfected BALB/c strain. American colonies of the BALB/c strain normally have an extremely low tumor incidence, and MMTV cannot be found in the milk of lactating females or in the occasional mammary tumor (Nandi and McGrath, 1973). Virtually all C3H females produce milk-borne virus [termed MMTV(C3H)] and develop mammary tumors; the incidence of tumors in the BALB/cfC3H mice is correspondingly high. Hence tumor development in the foster-nursed strain is dependent upon the acquisition of MMTV(C3H) from the C3H female.

Our central findings indicate first, that restriction endonucleases can distinguish proviral DNA introduced by infection with MMTV(C3H) from viral DNA endogenous to BALB/c mice; second, that mam-

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mary tumors and glands from BALB/cfC3H mice are infected with MMTV(C3H), whereas livers are uninfected; third, that a limited region of viral DNA probably emcompasses the points of attachment to cellular DNA, suggesting that the integrative process produces a provirus approximately colinear with viral RNA; fourth, that a large number of sites in cellular DNA can accommodate a newly acquired provirus; and finally, that each mammary tumor is composed of cells derived principally from one or a few of the many infected mammary gland cells.

Results

Strategies for Analysis of MMTV Proviral DNA with Restriction Endonucleases

To use restriction endonucleases in an analysis of MMTV-specific DNA acquired by infected tissues of BALB/cfC3H mice, we first prepared a physical map of the recognition sites for various enzymes within the DNA of MMTV(C3H). We also needed to determine the nature of the fragments containing endogenous MMTV-specific DNA (Varmus et al., 1972) generated by cleavage of DNA from uninfected BALB/c mice with the same enzymes.

Restriction sites within the DNA of MMTV(C3H) were mapped using methods recently described for similar studies with the DNA of MMTV(GR) (Shank et al., 1978). Linear and circular forms of unintegrated viral DNA were prepared from mink lung cells chronically infected with MMTV(C3H). Products of endonuclease digestion were detected by hybridization with MMTV-specific ^{32}P-cDNA after electrophoresis in agarose gels and transfer to nitrocellulose filters (Southern, 1975). Of the twelve restriction endonucleases tested, ten yielded identical products after incubation with either MMTV(C3H) DNA or MMTV(GR) DNA; the two exceptions were Xho I, which recognized one additional site in MMTV(C3H) DNA, and Sac I, which had an additional site in MMTV(GR) DNA (J. Majors, personal communication). The similarity with respect to recognition sites is consistent with the high degree of homology between the RNA genomes of MMTV(GR) and MMTV(C3H), as demonstrated by competition hybridization tests (Ringold et al., 1976). The two differences reconfirm that these virus strains—known to differ in biological attributes (Nandi and McGrath, 1973; Bentvelzen, 1974) and genomic sequence (Friedrich et al., 1976)—are, in fact, distinguishable isolates; the homogeneity of the digestion products for each virus also argues that the strains are genetically homogeneous.

Five of the twelve enzymes tested were used in the ensuing studies: Eco RI, which cleaves MMTV(C3H) DNA once; Pst I, which cleaves 5 times; Sac I and Bam HI, which cleave at two sites each; and Hpa I, which does not cleave MMTV(C3H) DNA. The locations of the sites for these enzymes on a DNA provirus are shown in Figure 1. In this illustration, the provirus is assumed to be co-extensive with the linear form of unintegrated viral DNA and hence approximately colinear with viral RNA. As shown below, Pst I, Bam HI and Sac I digestions of DNA from MMTV-infected cells yield internal fragments which distinguish acquired proviruses from endogenous proviruses and are therefore useful for assessing whether a tissue is infected with MMTV(C3H). In addition, Pst I and Sac I are particularly useful for determining the region of the viral DNA in which recombination with the host DNA has occurred. Eco RI and Bam HI are particularly useful for defining the number of sites in the host DNA which can accommodate an acquired provirus; each copy of DNA should yield two fragments whose size is dependent upon both the position of the restriction site within the provirus and the distance from the site of integration to a recognition site for Eco RI or Bam HI in host DNA.

Acquired Proviruses Can Be Distinguished from Endogenous Proviral DNA and Are Colinear with Unintegrated Linear DNA

We first tested DNA from BALB/cfC3H mammary tumors with Pst I to determine whether our techniques were adequate for detection of MMTV(C3H) proviruses in the face of endogenous viral DNA. In these experiments, we also asked whether a specific region of newly acquired proviruses was linked to cell DNA. An analysis of the kinetics of annealing of radioactive viral cDNA to an excess of unlabeled cellular DNA (Table 1; Morris et al., 1977) has shown that mammary tumors from BALB/

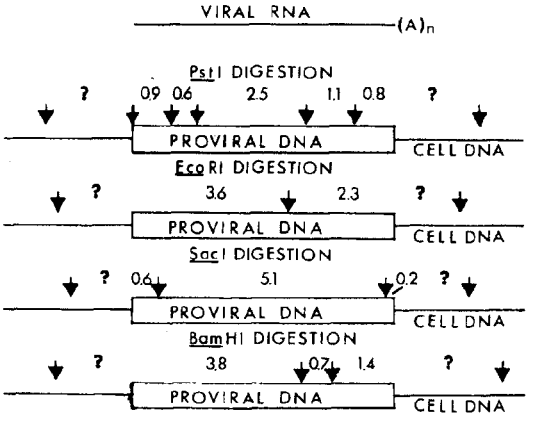

Figure 1. Schematic Diagram of Restriction Endonuclease Cleavage Sites in MMTV Proviral DNA

Arrows indicate restriction sites in proviral and cellular DNA. The molecular weights of viral fragments are indicated above each molecule in units of 10^6 daltons.

Table 1. Cot Analysis of MMTV Sequences in DNA from Various Mouse Tissues

Origin of DNA	$Cot_{1/2}$[a]	Copies per Diploid Cell[b]
BALB/c		
Pooled normal organs	290	7
Lactating mammary glands		
Primiparous	250	8
Multiparous	280	7
Mammary tumor	260	8
BALB/cfC3H		
Pooled normal organs	280	7
Lactating mammary glands		
Primiparous	260	7
	285	7
Multiparous	220	9
	275	7
	325	6
Mammary tumors[c]		
24 (B)	80	25
25 (H)	250	10
26	83	22
27	200	11
28A (D)	100	23
28B	160	13
28C (F)	180	12
29A (E)	150	13
29B	200	10
39	150	13

[a] Cot units are mole·sec/l.

[b] The number of copies of MMTV proviral DNA per cell was determined using DNA-DNA hybridization between viral-specific ^{3}H-cDNA and DNA extracted from mouse tissues. Hybridization conditions were as described in Experimental Procedures, and copy numbers were determined by comparison of the rate of annealing of viral ^{3}H-cDNA and mouse unique sequence ^{14}C-DNA ($Cot_{1/2}$ ≈ 2100 mole·sec/l) as described previously (Morris et al., 1977).

[c] Tumors are designated by animal number, and in cases where more than one tumor was obtained from the same animal, a capital letter is added to distinguish each individual tumor. The corresponding lane in Figure 8 is indicated by the letter in parentheses.

cfC3H mice harbor a greater number of copies of MMTV DNA (10–25 per diploid cell) than do normal BALB/c organs (~7 per cell). Comparisons of the products of Pst I digestion of DNA from BALB/cfC3H mammary tumors (28A, 28B and 28C; Table 1) and from normal livers revealed significant differences in MMTV-specific fragments. We observed five viral-specific fragments (3.3, 3.1, 1.1, 0.9 and 0.5 × 10^6 daltons) in digestions of normal BALB/c liver DNA (Figure 2, lane F). Pst I digestion

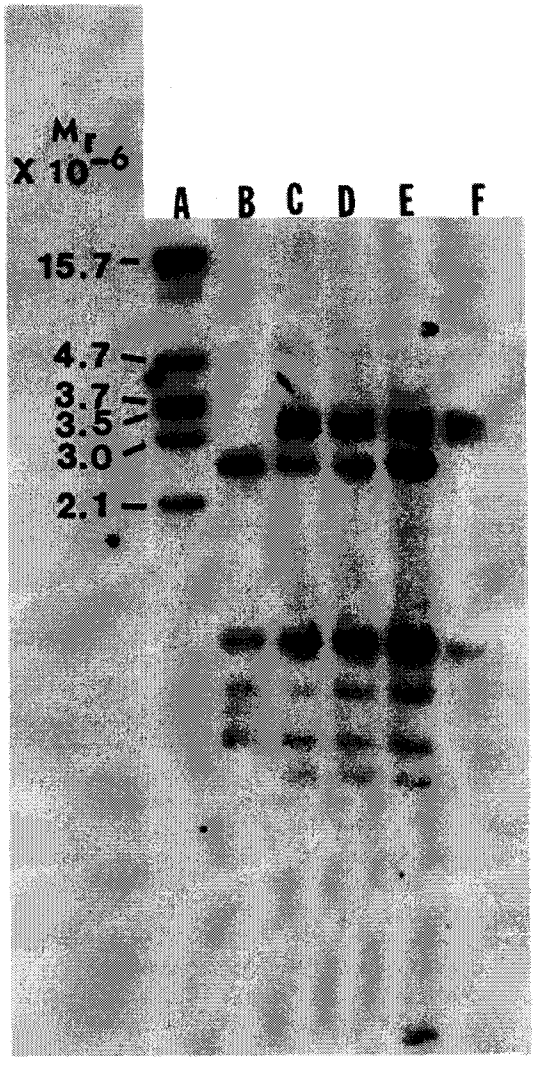

Figure 2. Demonstration That Digestion with Pst I Distinguishes Acquired from Endogenous Proviral DNA in BALB/cfC3H Mouse Tissues

10 μg samples of cellular DNA were digested with the restriction enzyme Pst I and viral-specific fragments were electrophoresed on 0.8% agarose gels. DNA was transferred to nitrocellulose sheets by the method of Southern and hybridized with $cDNA_{vep}$ as described in Experimental Procedures. For comparison, unintegrated MMTV(C3H) linear DNA, isolated from the cytoplasm of MMTV(C3H)-infected mink lung cells, was also treated with Pst I; Eco RI fragments of ^{32}P-labeled lambda bacteriophage DNA were included as molecular weight standards in a parallel lane. (A) Eco RI digest of ^{32}P lambda phage DNA; (B) Pst I digest of linear MMTV(C3H) DNA; (C–E) Pst I digests of DNA from BALB/cfC3H mammary tumors 28A, 28B and 28C, respectively (see Table 1); (F) Pst I digest of DNA from BALB/c liver.

of tumor DNA revealed two additional viral-specific fragments (2.5 and 0.6 × 10^6 daltons) unique to tumor DNA (Figure 2, lanes C–E). Both fragments appearing only in digests of tumor DNA are present in digests of the linear form of unintegrated DNA

(Figure 2, lane B) and have been mapped to internal positions in the linear form (Figure 1; Shank et al., 1978). The intensities of the bands representing fragments of 1.1 and 0.9 × 10^6 daltons were increased relative to the bands at 3.3 and 3.1 × 10^6 daltons in the digests of tumor (as opposed to liver) DNA. The augmented intensities suggested that these fragments, which are common to digests of both unintegrated viral DNA and endogenous viral DNA in BALB/c liver, are also common to acquired and endogenous viral DNA in mammary tumors. The generation of these two fragments from acquired proviruses again implies that they usually occupy internal positions in integrated as well as unintegrated DNA (Shank et al., 1978). An additional fragment of 0.8 × 10^6 daltons, derived from the end of unintegrated viral DNA, was not present in digests on tumor DNA, suggesting that the region defined by this fragment was always linked to cellular DNA.

To show unequivocally that all the major Pst I fragments other than the 0.8 × 10^6 dalton fragment could be obtained by digestion of acquired proviruses, we exploited our observation that Hpa I cleaves endogenous MMTV DNA to yield fragments of 4.4 and 4.2 × 10^6 daltons (Figure 3A, lane a; J. C. Cohen and H. E. Varmus, unpublished observation) but does not cleave MMTV(C3H) DNA (Shank et al., 1978). By separating DNA of greater than genome size (~6 × 10^6 daltons) from smaller DNA in an Hpa I digestion of mammary tumor DNA (see the legend to Figure 3), we effectively separate acquired form endogenous proviral DNA (Figure 3B, lane a). Redigestion of high molecular weight Hpa I fragments with Pst I yielded fragments of 2.5, 1.1, 0.9 and 0.6 × 10^6 daltons (Figure 3B, lane b), but none of the fragments unique to endogenous proviral DNA (3.3, 3.1 × 10^6 daltons; Figure 3B, lane c). The 0.5 Pst I fragment includes the Hpa I site unique to endogenous proviral DNA and thus would not be seen in a double digestion (our unpublished observation).

This experiment with Pst I clearly distinguishes acquired from endogenous provirus and appears to argue that a region encompassed by two Pst I sites, 0.8 × 10^6 daltons apart on circular DNA, is specifically linked to host cell DNA. It could be contended, however, that this region is simply absent from most or all acquired proviruses of mammary tumors, and that integration occurs randomly within viral DNA. We have countered this argument by first making use of the fact that the enzyme Sac I cleaves MMTV(C3H) DNA near both ends of unintegrated linear DNA (0.6 daltons from the left-hand end and 0.2 × 10^6 daltons from the right-hand end; Figure 1; J. Majors, personal communication). Hence it cuts within the region represented by the 0.8 Pst I fragment. If this region is represented within proviral DNA, digestion of acquired proviruses with Sac I will produce a fragment of 5.1 × 10^6 daltons. Digestion of BALB/c DNA with Sac I generates a fragment of 5.0 × 10^6 daltons in addition to fragments of molecular weight 7.0, 5.8, 4.3, 3.0 and 1.2 × 10^6 daltons (Figure 4, lane A); thus Sac I is not useful for sensitive detection of acquired proviruses. In digests of DNA from mammary tumors with multiple copies of newly acquired proviral DNA, however there is an obvious increase in relative intensity and a broadening of the band representing viral-specific fragments of 5.1 × 10^6 daltons (Figure 4, lanes C-E). No other new bands are observed consistently, indicating that most if not all acquired proviruses retain the region represented by the Pst I fragment of 0.8 × 10^6 daltons. Moreover, the results further define the region of viral DNA linked to cell DNA: the point of attachment must be located in most cases between the Sac I site 0.2 × 10^6 daltons from the right-hand end of linear DNA and the Pst I site within 0.1 × 10^6 daltons of the left-hand end. Thus acquired proviruses appear

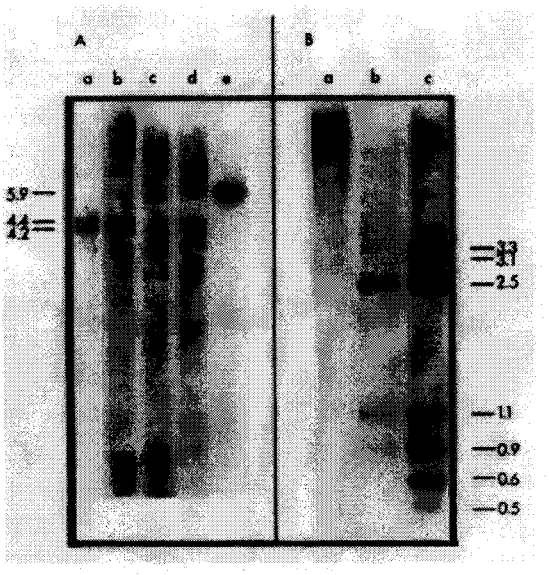

Figure 3. Separation of Endogenous from Acquired Proviruses by Hpa I Digestion

(A) DNA (10 μg) was digested with Hpa I and electrophoresed as described in the legend to Figure 2. (a) BALB/c liver; (b-d) BALB/cfC3H tumors 28A, 28B and 28C (see Table 1), respectively; (e) unintegrated MMTV (C3H) linear DNA. (B) 20 μg of BALB/cfC3H tumor (28A) DNA were digested with Hpa I. After electrophoresis in a 0.8% agarose gel, that fraction of the cell containing DNA >5.9 × 10^6 daltons was removed and dissolved in 7 M $NaClO_4$, and the DNA was recovered by hydroxyapatite chromatography. This DNA was either reelectrophoresed without prior digestion (a) or with prior Pst I digestion (b). Lane (c) shows a Pst I digest of a BALB/cfC3H tumor DNA.

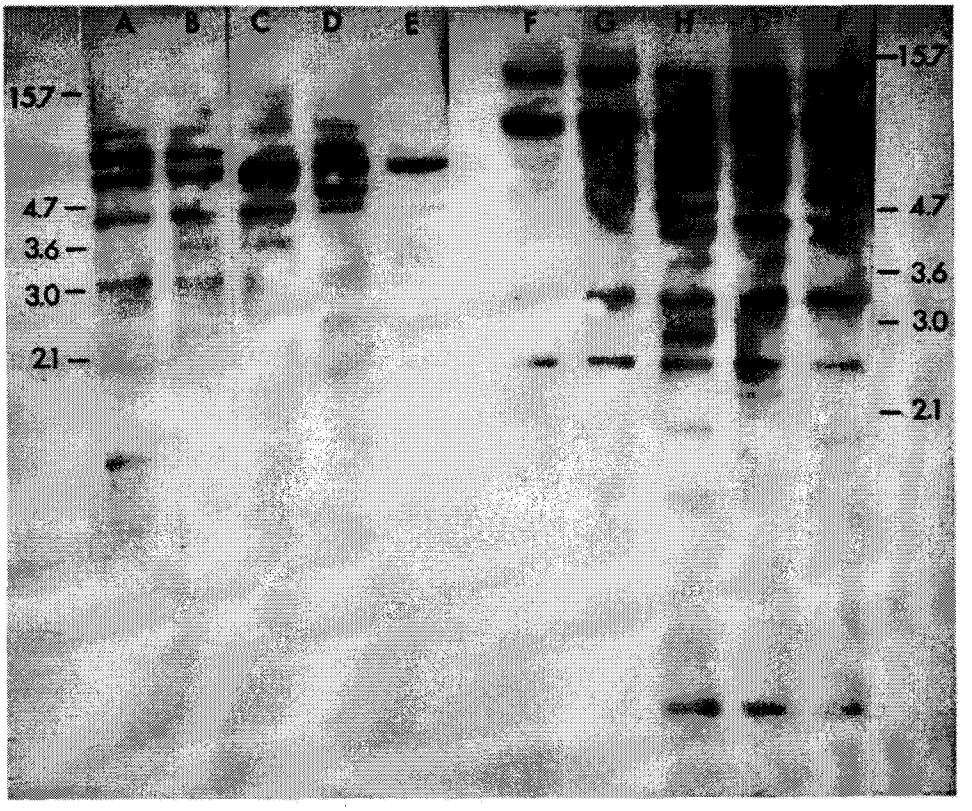

Figure 4. Comparison of Sac I and Bam HI Digests of Uninfected and Infected Tissues of BALB/cfC3H Mice

DNA from livers and lactating mammary glands obtained 10-20 days postpartum and from mammary tumors (28A, 28B, 28C; Table 1) was digested with either Sac I, lanes (A-E), or Bam HI, lanes (F-J), and analyzed on a 0.8% agarose gel as described in the legend to Figure 2. The molecular weights × 10^{-6} for Eco I-digested bacteriophage lambda DNA fragment markers are indicated on the left for Sac I digestion and on the right for Bam HI digestions. Sac I-cleaved DNA from BALB/cfC3H liver (A), mammary gland (B) and tumor 28A (C), 28B (D) and 28C (E). Digestions with Bam HI of DNA from BALB/cfC3H liver (F), mammary gland (G) and tumor 28A (H), 28B (I) and 28C (J).

colinear with unintegrated viral DNA. In the second experiment, digestion of DNA from three tumors with Bam HI produces, as expected, the internal fragment of 0.7 × 10^6 daltons which was common to all digestions (Figure 4, lanes H-J). If integration occurred randomly, viral DNA from most or all tumors bearing many copies of viral DNA would be expected to yield a Bam HI fragment of 5.2 × 10^6 daltons from proviruses attached to cellular DNA within the 0.7 × 10^6 Bam HI fragment. Since the 0.7 × 10^6 dalton fragment is unique to acquired proviruses (compare lanes F and H, Figure 4), it serves as a useful marker for infection.

The experiments shown in Figures 2, 3 and 4 suggest the following conclusions: first, that the appearance of Pst I (2.5 and 0.6 × 10^6 daltons) and Bam HI (0.7 × 10^6 daltons) fragments unique to acquired proviruses can be used to monitor infection of mouse cells despite the presence of endogenous viral-specific DNA; and second, that a relatively limited region of viral DNA defined by Sac I and Pst I recognition sites approximately 0.2 × 10^6 daltons apart on the unintegrated circular DNA appears to include the region at which the provirus is linked to cellular DNA.

Detection of Acquired Proviruses in Lactating Mammary Glands of MMTV-Infected Mice

Having shown that restriction endonucleases could be used to assay tissues for the presence of MMTV proviruses acquired by infection, we next asked whether new proviruses could be detected in tissues from nontumor-bearing BALB/cfC3H mice exposed to virus by nursing on infected females. These experiments were designed to address several questions: Does the virus demonstrate tissue tropism? Does it infect a sufficient number of cells in target tissue to allow detection by biochemical techniques? If so, are there significant differences in the organization or sites of integration of proviral DNA in normal and in tumor tissues?

The tissue most likely to contain a significant

number of MMTV-infected cells is the mammary gland, since virus can be found at high titers in the milk of infected mice which have not yet developed tumors (Nandi and McGrath, 1973). We initially attempted to estimate whether significant numbers of mammary gland cells from BALB/cfC3H mice were infected by measuring the rate of annealing of MMTV cDNA to DNA from lactating mammary glands; however, we could measure no difference in the rates of annealing of mammary gland DNA and DNA from normal BALB/c organs (Table 1). Since a small increment in number of copies per cell might not have been detectable in this test, we attempted to determine the proportion of infected cells by looking for fragments specific for the MMTV(C3H) provirus in Pst I digests of DNA from BALB/cfC3H lactating mammary glands, as illustrated above with mammary tumors (Figure 2). An MMTV(C3H)-specific fragment of 2.5×10^6 daltons was readily detected in Pst I digests (Figure 5, lane D) and a fragment of 0.6×10^6 daltons was also observed, indicating that acquired proviruses were present in this tissue. This finding was confirmed by detection of a 0.7×10^6 dalton fragment unique to acquired proviruses in Bam HI digests of BALB/cfC3H mammary gland DNA (Figure 4, lane G). Again the observed viral fragments in Pst I and Bam HI digests of lactating mammary gland DNA are consistent with the hypothesis that the point of linkage of viral DNA to cellular DNA is within a limited region of the viral DNA. New MMTV proviruses were not detectable in the DNA from livers of the foster-nursed animals (Figure 5, lane C; Figure 4, lane F), suggesting that the liver is not a target organ for MMTV. In addition, the pattern of viral-specific fragments was the same in the DNA from both mammary glands and livers of BALB/c mice which were not foster-nursed (Figure 5, lanes A and B), further documenting the relationship between virus infection and the appearance of Pst I fragments specific for MMTV(C3H) in glandular DNA.

Figure 5. Detection of Acquired MMTV Proviruses in the Mammary Glands of BALB/cfC3H Mice

Lactating mammary glands and livers from females 10–20 days postpartum were obtained and DNA was extracted for digestion with Pst I. 10 μg of DNA from each sample were analyzed as described in the legend to Figure 2. (A) BALB/c liver, (B) BALB/c lactating mammary gland, (C) BALB/cfC3H liver, (D) BALB/cfC3H lactating mammary gland.

To estimate the average number of copies of MMTV(C3H) DNA per mammary gland—and thereby the proportion of infected cells in the glands—we calibrated the assay shown in Figure 5 with the reconstruction experiment illustrated in Figure 6. Varying proportions of DNA from BALB/c livers and from a BALB/cfC3H tumor containing about 20 copies of MMTV(C3H) provirus were prepared, digested with Pst I and analyzed in parallel with the experiments in Figure 5. A 1:10 mixture of tumor DNA:liver DNA yielded results similar to those obtained with DNA from the lactating mammary gland (Figure 5). Thus the mammary gland cells contained an average of 1–2 copies of new provirus per diploid cell. We cannot, of course, determine from this analysis how uniformly the proviruses were distributed among the glandular cells. It is improbable, however that the infected cell contained more copies of viral DNA than we have measured in mammary tumor cells (Table 1). Hence we would suppose that a minimum of 5–20% of the cells are infected, but the proportion may be higher. Tests for viral antigen using the horseradish peroxidase method are consistent with these conclusions, demonstrating that generally 10–75% of acinar cells in a BALB/cfC3H lactating mammary gland are productively infected (unpublished data of R. Cardiff).

In the experiment shown in Figures 4 and 5, the lactating mammary gland was obtained from an animal in its third period of lactation. In view of evidence that the incidence of mammary tumors

may increase with forced breeding and multiple periods of lactation (Nandi and McGrath, 1973), we have examined the DNA from mammary glands of BALB/cfC3H animals at different periods of lactation. Digestion with Pst I demonstrated acquired proviruses in glands from an animal in the fifth and another animal in the third lactation, in concentrations similar to those found in the previous experiment; acquired proviruses, however, were not detectable in three other animals in their first, second and fourth lactation (data not shown). Further experiments will be required to evaluate the effects of repeated lactation upon virus spread.

Many Sites in the DNA of Mammary Glands Can Accommodate an MMTV Provirus

Having demonstrated that a significant proportion of cells in some lactating mammary glands are infected with MMTV(C3H), we asked whether the MMTV(C3H) proviruses were integrated preferentially at certain sites in the host genome. Since the experiments with Pst I, Bam HI and Sac I (Figures 2, 3 and 4) indicated that the MMTV(C3H) proviruses had a similar internal order (as drawn in Figure 1), cleavage with either Eco RI or Bam HI should yield two fragments containing viral sequences which are specific for each provirus, with the size of the pieces dependent upon the distance from the integration site to either an Eco RI or Bam HI site in the surrounding cellular DNA (see Figure 1). (Cleavage of Bam HI would, of course, also result in a fragment of 0.7×10^6 daltons common to all the acquired proviruses.) Similar fragments would occur with Pst I and Sac I, but only a small amount of viral DNA ($<0.8 \times 10^6$ daltons) would be present in DNA fragments containing cellular sequences, since sites for Pst I and Sac I (Figure 1) occur at or near the ends of the proviral DNA. If many acquired proviruses (for example, 5–10% or more) were located in similar positions in the DNA of infected mammary gland cells, we would expect to see a discrete band (or bands) of viral-specific fragments in addition to those representing Eco RI and Bam HI fragments of endogenous viral DNA. Analysis of the Eco RI and Bam HI digests of DNA from BALB/cfC3H lactating mammary glands (Figure 4, lane G; Figure 7, lane D), however, revealed no bands other than those also found in similar digests of BALB/c liver DNA (Figure 4, lane F; Figure 7, lane A). This analysis cannot, of course, exclude the unlikely possibility that fragments of homogeneous size might have been obscured by similarly sized fragments of endogenous MMTV DNA upon digestion with two restriction endonucleases; moreover, integration of less than 5–10% of proviruses into similar sites might have been below the level of sensitivity of this assay. We tentatively conclude from this experiment that multiple sites in the genome of mammary gland cells must be able to accommodate an MMTV provirus.

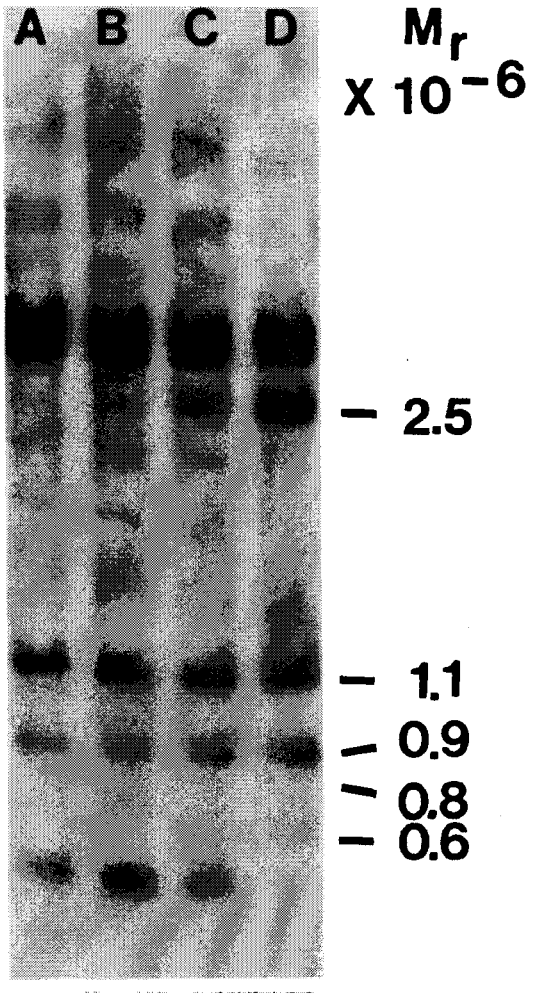

Figure 6. Reconstruction Experiment with BALB/cfC3H Liver and Tumor DNAs

DNAs from liver and mammary tumor (26; Table 1) were mixed in differing proportions and digested with Pst I prior to analysis as described in the legend to Figure 2. Tumor DNA to liver DNA ratios of 1:40 (A), 1:20, (B) 1:10 (C) and 1:5 (D) were used.

We further explored the possibility that newly acquired proviruses were integrated at multiple sites in the host genome by subjecting the DNA from a large number of BALB/cfC3H tumors to digestion with Eco RI. DNA samples from several mammary tumors, which may have from 3 to 18 additional copies of MMTV DNA per diploid cell (Table 1), were digested with Eco RI and Bam HI and analyzed by gel electrophoresis and hybridization (Figures 4 and 8). The most striking finding was the appearance of several different viral-specific fragments in the digests of DNA from each tumor, even from multiple tumors from the same animal (Figure 4, lanes H–J; Figure 8, lanes B and

C, D and F), indicating that many sites in host DNA were occupied by acquired proviruses. Correlation of copy number obtained by DNA-DNA hybridization kinetics (Table 1) was possible to some extent. For example, DNA from tumor 25, with a low number of new proviruses detected by solution hybridization, yielded only a few new Eco RI fragments in addition to those characteristic of the endogenous virus (Figure 8, lane H), while DNA from tumor 28A, with multiple copies of acquired MMTV DNA, yielded many new fragments (Figure 4, lane H; Figure 8, lane D). Furthermore, the fragments obtained by cleavage of DNA from BALB/c liver with Eco RI or Bam HI (Figure 4, lane F; Figure 8, lane A) were also present in the digests of tumor DNA (Figure 4, lanes H–J; Figure 8, lanes B–M), suggesting that the acquired proviruses did not integrate into endogenous MMTV sequences. In addition, few if any new bands of ~6 × 10^6 daltons were observed in Eco RI digestions, indicating that the tumors with multiple new copies of viral DNA rarely contained those copies in tandem. (Such an arrangement would yield fragments of unit length upon cleavage by an enzyme with one recognition site per provirus.)

Although the location of the Eco RI site within a provirus of normal size (see Figure 1) predicts that no new fragments of less than 2.3 × 10^6 daltons can result from digestion with Eco RI, a few fragments of smaller size were observed. Three or four of these appeer to be common to all tumors *and* to normal cellular DNA, although the intensities of observed bands vary considerably both within and among experiments (for example, compare Eco RI digestions in Figures 7, 8 and 9). Furthermore, digestion of tumor DNA with Sac I or Bam HI did not yield similar sets of fragments (Figure 4). These fragments may contain a minor component of endogenous viral DNA or cellular sequences detected by a minor nonviral contaminant in the labeled hybridization reagent; we cannot explain the variable intensities of these bands, however, and we have not yet attempted to distinguish among the several possible explanations for them.

Since many different sites in the cellular genome are occupied by MMTV(C3H) proviruses in the various tumors as well as in the lactating mammary glands, it would appear that incoming viral DNA molecules can integrate at many positions in cellular DNA, perhaps at random. In any single tumor, however, several discrete new bands were observed. This apparent anomaly could be explained if each tumor consisted principally of cells derived from only a few of the many infected mammary gland cells; most of the cells would then bear some of their proviruses at the same position in host DNA, thereby yielding discrete bands in our analyses. The diffuse background of annealed viral cDNA observed in the analysis of Eco RI digests of DNA from some tumors (for example, lanes B, D and L in Figure 8) could represent proviral DNA acquired and inserted at many sites during propagation of the tumor or proviral DNA in a subset of tumor cells derived from a large number of precursor cells. We have not rigorously excluded the possibility, however, that the tumors are composed of descendents of several independently infected cells, or that the discrete Eco RI fragments derived from the DNA in each tumor are consequent to integration into some of a limited number of sites which promote the oncogenic activity of the virus. We have attempted to judge the likelihood of these other interpretations in two ways: by analysis of DNA from several secondary tumors derived by transplantation of small samples of primary tumors, and by analysis of a pool of DNA from a large number of primary tumors.

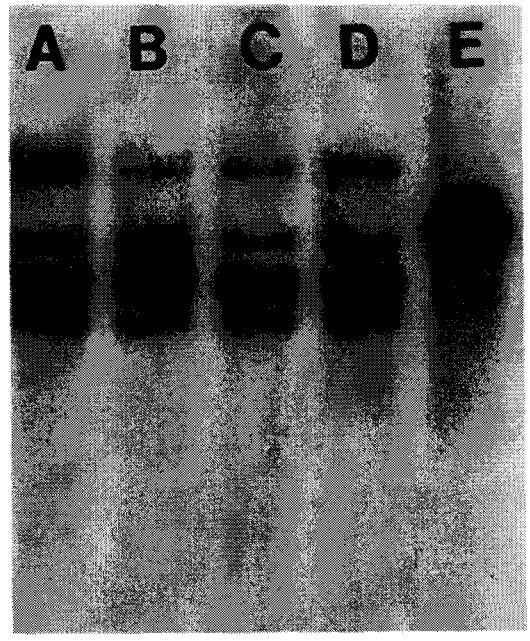

Figure 7. Comparison of Eco RI Digestions of Liver and Mammary Gland DNAs

Tissues were obtained 10–20 days postpartum; DNA was extracted and digested with Eco RI as described in the legend to Figure 2. BALB/c liver (A) and mammary gland (B); BALB/cfC3H liver (C) and mammary gland (D); MMTV(C3H) unintegrated linear DNA (E).

We first challenged the notion that a mammary tumor is composed principally of cells derived from one or a few of the many infected mammary gland cells by examining Eco RI digests of DNA from pieces of a primary tumor and from several transplanted tumors derived from each piece of the spontaneous tumor (Figure 9). The transplantation

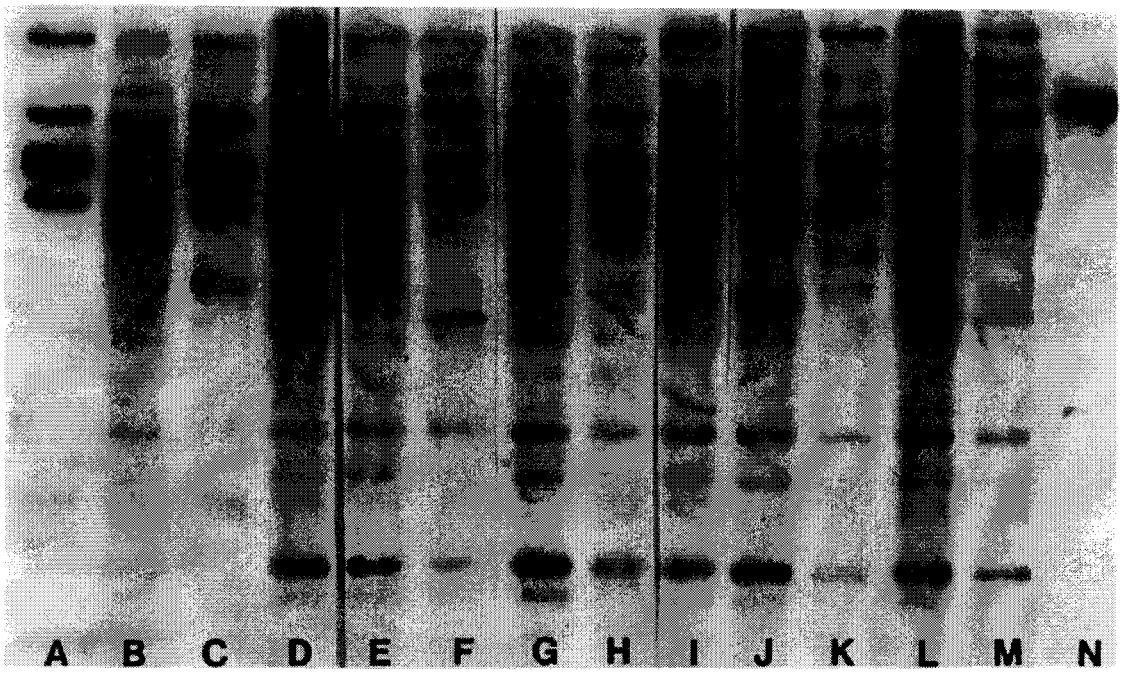

Figure 8. Comparison of Integrated MMTV DNA in Mammary Tumors from BALB/cfC3H Mice

DNA (10 μg) from the various tissues was digested with Eco RI, electrophoresed in a 0.8% agarose gel and annealed with MMTV cDNA after transfer to nitrocellulose filter sheets. (A) BALB/c liver; (B–M) BALB/cfC3H mammary tumors (see Table 1); (N) undigested linear MMTV(C3H) DNA.

procedure was designed to optimize the possibility of generating secondary tumors with differing properties: small samples (2 x 2 mm) were removed from widely separated regions in each segment of the primary tumor and transplanted into the fat pad of separate recipient BALB/c mice. DNA from all of the transplanted tumors yielded the same set of acquired viral-specific Eco RI fragments (indicated by arrows) as did DNA from the original tumor (Figure 9). Hence this experiment supports the idea that the tumors are composed of a relatively homogeneous population of cells, since we failed to observe differences among integration sites for viral DNA in the transplanted tumors.

We next attempted to determine whether some of the MMTV(C3H) proviruses in tumor DNA were situated in a limited number of sites. Samples (1 μg) of DNA from the twelve tumors studied (Figure 8) were mixed after cleavage with Eco RI in an effort to demonstrate fragments common to some of the tumors; these would appear as bands imposed upon a diffuse background of annealing, and they would presumably reflect a higher than average occupancy of selected sites in the host DNA. Examination of the mixture (Figure 10), however, revealed only those discrete bands of Eco RI fragments which co-migrate with Eco RI fragments derived from uninfected BALB/c DNA; apparently the acquired proviruses were located at a sufficiently large number of sites that no preferred sites could be identified by this technique.

Discussion

Documenting Infection with Restriction Enzymes

This report has focused upon a single step in the replication of MMTV in its natural host: the integration of viral DNA into the cellular genome following infection of mice with milk-borne virus. We have shown that restriction endonucleases can be used effectively to differentiate proviruses acquired by infection from those endogenous to the cells in both mammary tumors and lactating mammary glands. Pst I, an enzyme which cleaves MMTV(C3H) DNA at several sites, has proved to be particularly useful for this task, since it generates a relatively large, unique fragment which is derived from internal regions of the provirus and is specific for the provirus of the infecting virus strains. Thus Pst I allows detection of new viral DNA even when the proviruses are positioned at many sites throughout the cellular genome, because the critical digestion products are not linked to cellular DNA. The identification of restriction endonuclease fragments specific for the infecting provirus should not, however, be interpreted to mean that infection by

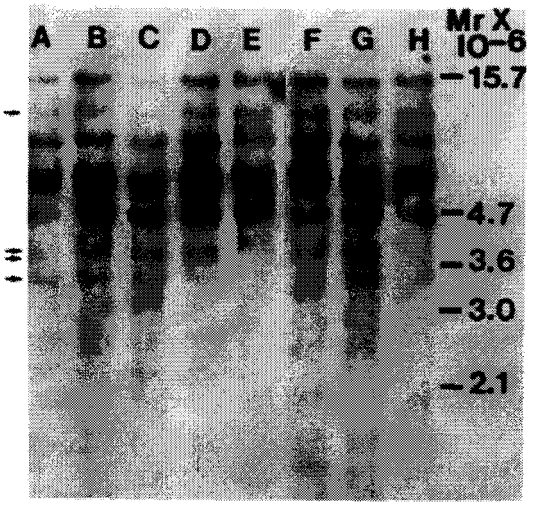

Figure 9. Invariance of Eco RI Fragments of Proviral DNA in Explants of a Tumor from a BALB/cfC3H Strain

A single tumor from a BALB/cfC3H mouse was halved, and 2 x 2 mm fragments from each half were transplanted into the fat pad of BALB/c females. 10 μg of DNA from tumors developing from the transplanted tissue or from the halves of the original tumors were treated with Eco RI and electrophoresed on 0.8% agarose gels. (A and F) Halves of primary tumor; (B–E) secondary tumors derived from tissue shown in lane (A); (G and H) secondary tumors derived from tissue shown in lane (F). Arrows indicate fragments acquired by infection with exogenous virus and common to all tumors. Eco RI fragments of lambda phage DNA (not shown) were used as molecular weight standards.

MMTV(C3H) introduces important genetic elements not present in the endogenous proviruses. Minor base changes or rearrangements could readily account for the enzymatic distinctions which we have made here between the endogenous and acquired proviruses.

Thus far our tests for acquired proviruses have confirmed the general assumption that mammary tumors and lactating mammary glands of BALB/cfC3H mice are infected since they produce virus, whereas liver cells are not (Figures 4 and 5). MMTV-induced tumors have previously been shown, by conventional molecular hybridization in solution, to contain either additional copies of viral DNA (Morris et al., 1977) or viral sequences which could not be detected in liver DNA (Michalides and Schlom, 1975; Drohan et al., 1977). It will now be of interest to attempt to follow the natural route of infection by milk-borne virus, particularly to determine whether leukocytes (with which virus transiently associates) are infected, to analyze the spread of infection in mammary glands under various physiological conditions and to search for other target organs for the virus.

A Specific Region of Viral DNA Joined to Cellular DNA

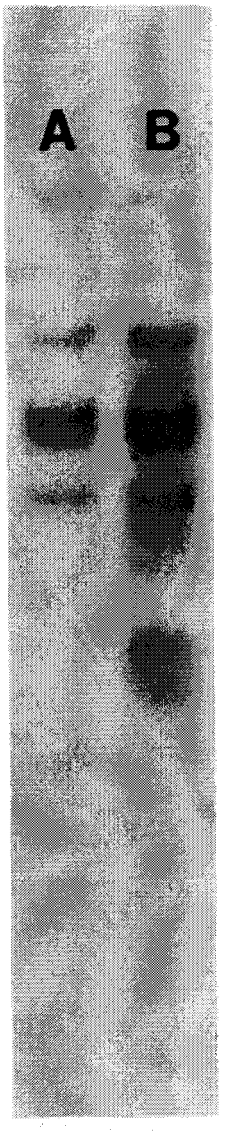

Figure 10. Examination of BALB/cfC3H Mammary Tumor DNAs for Preferred Sites of Integration

A mixture containing 1 μg of DNA from each of the tumors presented in Figure 8 was digested with Eco RI followed by analysis as described in the legend to Figure 2. (A) BALB/c liver DNA (12 μg); (B) BALB/cfC3H mammary tumor DNA mixture (12 μg).

I, Sac I and Bam HI are consistent with the proposal that a limited region of the viral DNA is preferentially joined to cellular DNA, indicating that the mechanism of integrative recombination has specificity for a site (or sites) in viral DNA. In the studies reported here, we have localized the region to 0.2 $\times$ 10^6 daltons encompassing the ends of unintegrated linear DNA; similar results have been obtained in studies of MMTV DNA in heterologous rat

that linear DNA is approximately colinear with viral RNA (Shank et al., 1978). Since viral RNA is presumably synthesized by transcription of proviral DNA, there would appear to be a requirement for integration of viral DNA in an orientation approximately colinear with viral RNA. Our results are consistent with that requirement. Similar studies of integration of the provirus of avian sarcoma virus (ASV) into cellular DNA indicate that a restricted region of the viral DNA within 50 nucleotides of each end of linear DNA is preferentially joined to cellular DNA (Hughes et al., 1978).

Many Sites in Host DNA Can Accommodate an MMTV Provirus

The use of specific regions of the viral DNA for integrative recombination distinguishes the integration of RNA tumor virus DNA from the integration of papovavirus DNA; the latter appears to occur at random sites on both viral and cellular genomes (Ketner and Kelley, 1976; Botchan, Topp and Sambrook, 1976). It might then have been predicted that we would also find specific sites in host DNA used for the integration of RNA tumor virus DNA. Thus far, however, we can conclude only that there are a large number of sites at which proviral DNA can be found. In each mammary tumor which we have studied, MMTV(C3H) DNA was located in multiple different sites. Furthermore, DNA pooled from several tumors and DNA from mammary glands containing a large number of independently infected cells failed to reveal new Eco RI fragments of discrete size that would be indicative of preferred integration sites. MMTV proviruses are also found at many different sites in the DNA of cloned MMTV-infected rat hepatoma cells (G. M. Ringold, personal communication). Neither set of experiments places clear limits on the number of sites available for acquired proviruses. On theoretical grounds, there could be as many as 10^9 sites (for instance, in the case of completely random integration); thus our lower limit for the number of available sites ($\sim 10^2$) does not preclude a still relatively small set of preferred sites. Identification of such sites will presumably require the use of restriction enzymes which cleave cellular DNA very close to the ends of the provirus or direct nucleotide sequencing of the sites of linkage of viral and cellular DNA.

Mammary Tumors Are a Select Subpopulation of Infected Cells

Although we have presented evidence favoring a large number of integration sites for MMTV DNA in cellular DNA, the DNA from each mammary tumor which we have examined yielded a well defined set of Eco RI and Bam HI fragments (Figures 4 and 8). These observations are most simply interpreted by postulating that each tumor consists of a subset of cells derived from one or a few of the many MMTV-infected mammary epithelial cells. We have provided partial confirmation of this hypothesis by showing that DNA from several secondary tumors grown by transplantation of primary tumors yielded the same MMTV-specific Eco RI fragments as did DNA from the primary tumor (Figure 9). It would obviously be preferable to examine this question by testing multiple clones derived from mammary tumors in tissue culture. In general, however, it is difficult to establish mammary carcinoma cells in culture, particularly as clones. Although several mammary tumor cell lines are available, most are not well suited for the proposed studies because they contain very high numbers of copies of MMTV DNA (our unpublished observation) or because they were established with cells pooled from multiple tumors (Yagi, 1973; Sarkar, Pomenti and Dion, 1977).

Is the Site of Integration of Biological Significance?

Since MMTV proviruses can be found at many sites in the host genome, it is of obvious interest to ask whether their location within the host genome has any impact upon viral gene expression or upon tumorigenesis. Ringold, Shank and Yamamoto (1978) have asked whether the site of integration of MMTV DNA in the genome of heterologous cells has a determining influence on the transcription of the provirus into RNA or on the regulation of transcription by glucocorticoid hormones. In the present context, we have attempted to determine whether the site of integration affects the probability that any one of the very large number of infected mammary epithelial cells will undergo progressive growth to form a clone of tumor cells. This issue is of particular interest, since no viral gene has been specifically associated with the oncogenic activity observed in vivo. Although we have found no evidence in the present study to suggest that the sites for integration of proviral DNA differ in tumor cells and infected cells from those of normal lactating mammary glands, more detailed studies of integration sites in tumors and phenotypically normal cells, coupled with a better definition of viral genes, will be required to evaluate this notion further.

Experimental Procedures

Cell Culture and Mouse Strains

Mink lung cells CCL64 (Valdya et al., 1976) were chronically infected as described previously (Ringold et al., 1977a) with MMTV(C3H) derived from the Mm5mt/ci mammary carcinoma cell line (Fine et al., 1974; virus provided by Frederick Cancer Research Center, Bethesda, Maryland). When these cells were used as a source for MMTV DNA, dexamethasone (10^{-6} M) was added 48 hr prior to the harvesting of cells to stimulate levels of viral

DNA (Ringold et al., 1978). Fractionation of cells into cytoplasmic and nuclear fractions was accomplished by treatment with the nonionic detergent Nonidet P40 (Borun, Scharff and Robbins, 1967).

BALB/c and BALB/cfC3H mouse strains were obtained from inbred colonies at Simonson Laboratories (Gilroy, California). The BALB/cfC3H substrain was infected by foster nursing BALB/c animals on a C3H female with subsequent generations nursed on their own mother and maintained as a separate colony. Mammary glands were removed from either primiparous or multiparous females at 2-3 weeks postpartum. Spontaneous tumors were isolated from breeding females within the colony.

DNA Extraction

Linear MMTV DNA was obtained from the cytoplasmic fraction of infected mink lung cells. After digestion of protein with pronase (1 mg/ml) in the presence of 1.0% SDS, the DNA was deproteinized with phenol-chloroform (2:1). Viral DNA was purified further by electrophoresis on 0.8% agarose gels (see below) and isolation of DNA from that portion of the gel to which DNA of $\sim 6 \times 10^6$ daltons migrated. The agarose was liquified using 7 M $NaClO_4$, 0.01 M sodium phosphate (pH 6.8) at 45°C. DNA was separated from contaminating agarose by chromatography on hydroxyapatite and precipitated with cetyl-trimethyl ammonium bromide in the presence of yeast RNA carrier.

Tissues samples were dispersed in DNA extraction buffer [0.02 M Tris-Cl (pH 8.1), 0.01 M EDTA, 0.1 M NaCl] using a motor-driven, teflon-glass dounce homogenizer. Self-digested pronase and SDS were added to a final concentration of 1 mg/ml and 1%, respectively, and the sample was incubated at 37°C for 12 hr. DNA was deproteinized twice with equal volumes of phenol-chloroform (2:1) followed by extensive dialysis against 5 mM Tris-Cl (pH 7.4), 0.1 mM EDTA. DNAs for DNA-DNA hybridization were denatured and fragmented by treatment with 0.3 N NaOH at 80°C for 2 hr.

Hybridization Reagents and Conditions

Labeled cDNA was synthesized in a reaction catalyzed by AMV DNA polymerase using oligomers of calf thymus DNA as primers (Goulian, 1968). Briefly, MMTV RNA (1-2 μg) isolated from virions purified from the Mm5mt/cl cell line was added to a reaction consisting of 50 mM Tris-Cl (pH 8.1); 2 mM dithiothreitol; 8 mM $MgCl_2$; 40 mM KCl; 100 μg/ml actinomycin D; 200 μM dGTP, dATP, TTP; 0.5-1.0 mCi α-^{32}P-dCTP (350 Ci/mmole; New England Nuclear); or 1-2 mCi ^{3}H-dCTP; 70 U AMV polymerase; and 1 mg primers in a 250 μl reaction. After incubation at 37°C for 45 min, the preparation was alkali-treated (0.6 N NaOH, 37°C, 2 hr), neutralized with HCl, chromatographed on a Sephadex G-75 column to remove unincorporated dCTP and precipitated with ethyl alcohol.

Annealing reactions between MMTV ^{3}H-cDNA (1000 cpm per sample) and unlabeled cellular DNAs (1-3 mg/ml) were performed in 0.6 M NaCl, 0.04 M Tris-Cl (pH 7.4) and 0.002 M EDTA at 68°C for varying times up to 280 hr. Mouse unique sequence ^{14}C DNA (500 cpm per sample) was included as an internal standard in each reaction (Morris et al., 1977). Samples were fractionated by hydroxyapatite chromatography, and the number of MMTV genome equivalents per cell was calculated as described previously (Morris et al., 1977).

Filter hybridization of DNA transferred from agarose gels onto nitrocellulose sheets (Southern, 1975) was performed in an annealing mixture consisting of 3 x SSC (SSC = 0.15 M NaCl, 0.015 M Na-citrate), 50% formamide, 0.05 M HEPES buffer (pH 7.0), 200 μg/ml yeast RNA, 20 μg/ml alkali-sheared and denatured salmon sperm DNA, 1-2 $\times 10^6$ cpm ^{32}P-cDNA and Denhardt's buffer (Denhardt, 1966; 0.02% each of bovine serum albumin, polyvinylpyrrolidone and ficoll). Filters were incubated at 41°C with the annealing mix without cDNA for 12 hr prior to annealing; annealing mix containing cDNA was then added for 48 hr. After incubation, the filters were washed in 2 x SSC at room temperature for 1 hr, incubated in 0.1 x SSC-0.1% SDS at 50°C, rinsed with 0.1 x SSC-0.1% SDS and 0.1 x SSC at room temperature, and air-dried. Filters were exposed at -70°C to Kodak RP-Royal X-Omat film in the presence of Dupont Cronex "Lightning Plus" screens (Swanstrom and Shank, 1978).

Digestion of DNA with Restriction Endonucleases

Restriction endonucleases Eco RI and Pst I were gifts from P. Greene and W. Brown; Hpa I, Sac I and Bam HI were obtained from New England BioLabs (Beverly, Massachusetts). Digestion with Eco RI was performed in 0.1 M Tris-Cl (pH 7.4), 0.05 M NaCl, 5 mM $MgCl_2$, 0.05% NP40. Pst I reaction buffer consisted of 6 mM Tris-Cl (pH 7.2), 6 mM $MgCl_2$, 50 mM NaCl, 6 mM 2-mercaptoethanol, 100 μg/ml gelatin. Hpa I digestions were performed in 10 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 6 mM KCl, 1 mM dithiothreitol. Sac I digestions were performed in 6 mM Tris-Cl (pH 7.4), 20 mM NaCl, 6 mM $MgCl_2$, 2 mM 2-mercaptoethanol. The reaction buffer for Bam HI contained 6 mM Tris-Cl (pH 7.9), 0.15 M NaCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol. Completeness of reactions was monitored by adding either lambda bacteriophage DNA (for Eco RI and Sac I) or form I of plasmid pBR313 DNA (for Pst I, Bam HI and Hpa I) to a portion of the digestion mixture. In all cases, the cleavages were shown to be complete by analysis of the marker DNAs by gel electrophoresis. In general, a 5-10 fold excess of enzyme was used in each digestion and reaction volumes were 0.1-0.3 ml.

Gel Electrophoresis and DNA Transfer

DNA samples digested with restriction endonucleases were electrophoresed in 0.8% agarose (Seakem) gels containing 0.04 M Tris-acetate (pH 8.15), 0.02 M Na-acetate, 0.018 M NaCl, 0.002 M EDTA (Helling, Goodman and Boyer, 1974). Bromophenol blue was added as a tracking dye and samples were electrophoresed at 40-70 V for a time sufficient for the dye to migrate 12-18 cm. Gels were stained with ethidium bromide (2.5 μg/ml) to visualize included marker DNAs (Eco RI-cleaved lambda bacteriophage DNA) and processed for transfer to nitrocellulose sheets using the methods described by Southern (1975). Briefly, gels were treated with alkali (0.5 N NaOH, 1.5 M NaCl) at room temperature for 1 hr, followed by neutralization with 0.5 M Tris-Cl (pH 7.0), 1.5 M NaCl for 3-5 hr. DNA was transferred by capillary action from the gel to the filter for 1-2 days using 6 x SSC, and the filters were then dried at 80°C in vacuo for 2 hr.

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